Relationship Between Homocysteine and Superoxide Dismutase in Homocystinuria
Possible Relevance to Cardiovascular Risk

David E.L. Wilcken, Xing Li Wang, Tetsuo Adachi, Hirokazu Hara, Natalia Duarte, Kathryn Green, Bridget Wilcken

Abstract—A modest homocysteine elevation is associated with an increased cardiovascular risk. Marked circulating homocysteine elevations occur in homocystinuria due to cystathionine β-synthase (CBS) deficiency, a disorder associated with a greatly enhanced cardiovascular risk. Lowering homocysteine levels reduces this risk significantly. Because homocysteine-induced oxidative damage may contribute to vascular changes and extracellular superoxide dismutase (EC-SOD) is an important antioxidant in vascular tissue, we assessed EC-SOD and homocysteine in patients with homocystinuria. We measured circulating EC-SOD, total homocysteine (free plus bound), and methionine levels during the treatment of 21 patients with homocystinuria, 18 due to CBS deficiency, aged 8 to 59 years, and 3 with remethylating defects. We measured total homocysteine by immunoassay, EC-SOD by ELISA, and methionine by amino acid analysis and assessed interindividual and intraindividual relationships. There was a significant, positive relationship between EC-SOD and total homocysteine. For the interindividual assessment, levels were highly correlated, \( r=0.746, N=21, P<0.0001 \). This relationship was maintained after taking into account intraindividual patient variation \( (r=0.607, N=62, P<0.0001) \). In 2 newly diagnosed CBS-deficient patients, treatment that lowered the markedly elevated pretreatment homocysteine level (from 337 to 72 and from 298 to 50 μmol/L) reduced the associated elevated EC-SOD in each by 50%. EC-SOD and methionine levels were unrelated \( (r=0.148, n=39, P=0.368) \). The positive relationship between circulating EC-SOD and homocysteine could represent a protective antioxidant response to homocysteine-induced oxidative damage and contribute to reducing cardiovascular risk in homocystinuric patients. EC-SOD levels may be relevant to the pathogenesis of vascular disease in other patient groups. (Arterioscler Thromb Vasc Biol. 2000;20:1199-1202.)

Key Words: homocysteine ■ superoxide dismutase ■ oxidative stress ■ vascular disease ■ cystathionine β-synthase deficiency

Data first presented in 1976 provided evidence for an association between a modest elevation of homocysteine and coronary disease,\(^1\) and, as reviewed recently,\(^2,3\) this association has been confirmed for the coronary, peripheral, and cerebral circulations in many subsequent studies. The hypothesis that a modest homocysteine elevation might contribute to the pathogenesis of vascular disease arose from the investigation of patients with homocystinuria, a recessively inherited disorder of methionine metabolism due to cystathionine β-synthase (CBS) deficiency, a disorder in which thromboembolic events are frequent. There was also recognition that the established cardiovascular risk factors could account for only part of the occurrence and severity of vascular disease in the general population. Other risk factors had to exist. In untreated patients with homocystinuria due to CBS deficiency, plasma homocysteine levels are extremely high, often >300 μmol/L of free plus bound homocysteine, and such patients are at high cardiovascular risk. In a worldwide review of 629 largely unrelated homocystinuric patients, Mudd and colleagues\(^4\) found that a vascular event had occurred in ∼50% before the age of 30 years. However, treatment to lower the elevated homocysteine level in homocystinuric patients by using vitamin B\(_6\) (pyridoxine), folic acid, vitamin B\(_{12}\) and, if necessary, trimethylglycine (betaine) markedly reduced cardiovascular risk in both pyridoxine-responsive\(^4,5\) and the more severe pyridoxine-nonresponsive patients, although homocysteine levels remained well above the normal range.\(^5\)

Mild hyperhomocysteinemia is associated with a significantly increased cardiovascular risk,\(^6\) and there is evidence for a dose response between level and risk, even within the normal range.\(^7\) Therefore, it seems surprising that patients...
with treated CBS deficiency and much higher circulating levels of homocysteine have such an apparently low risk of vascular complications.5

The mechanisms mediating homocysteine-induced vascular changes are not completely defined, but it is established that patients with homocystinuria (CBS deficiency) and those with homocysteine elevations from other causes (eg, folate or B₁₂ deficiency) have impaired endothelial function as evidenced by reduced nitric oxide–mediated vasodilatation.8,9 There is also in vitro evidence that homocysteine enhances smooth muscle cell proliferation10 and for a possible link between elevated homocysteine and oxidative damage.11 Superoxide radicals react avidly with nitric oxide to form peroxynitrite, a potent oxidant. Peroxynitrite is demonstrable in atherosclerotic lesions and has been identified as nitrotyrosine.12,13

An important component of the endogenous antioxidant defense opposing the deleterious vascular effects of free radicals is superoxide dismutase (SOD) present in the vascular wall. Of the 3 SOD isoenzymes, cytosolic Cu,Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD), >90% of interstitial SOD is EC-SOD, a copper- and zinc-containing glycoprotein. In addition, circulating EC-SOD is in equilibrium with the EC-SOD on the surface of the endothelium and bound to proteoglycan.12–14 We recently showed that low plasma EC-SOD levels are independently associated with a history of myocardial infarction and that these levels are lower in current smokers and higher in women than men,15 findings consistent with EC-SOD’s being protective in relation to coronary disease. To explore a possible role for SOD in a high-risk population with grossly elevated circulating homocysteine, we have now measured both of these variables at different stages during treatment of a group of patients with well-documented homocystinuria.

Methods

Patients

We studied 18 patients with homocystinuria due to CBS deficiency, 8 males and 10 females aged between 8 and 59 years (mean, 30 years), and 3 male patients with homocystinuria due to remethylating defects, aged 6 months, 18 years, and 35 years. All patients had normal renal function as assessed by plasma creatinine levels. Only 1 patient, a CBS-deficient man aged 38, smoked. Two patients had modestly elevated blood pressure, which was easily controlled with β-blocker therapy. None had a vascular event. Of the 18 CBS-deficient patients, 12 were pyridoxine nonresponsive as defined previously; ie, after a full pyridoxine treatment regimen, circulating total free homocysteine remained persistently >20 μmol/L after an overnight fast,5 ie, >75 μmol/L of total (free plus protein-bound) homocysteine. All 6 pyridoxine-responsive patients received 100 to 200 mg of pyridoxine and 5 mg of folic acid daily orally plus vitamin B₁₂ by injection according to B₁₂ levels; all 12 pyridoxine-nonresponsive patients received, in addition, oral betaine 6 to 9 g daily given in divided doses.8 Of the 3 patients with remethylating disorders, there were 2 with cobalamin C defects, and the other had a defect of methylenetetrahydrofolate reductase. All were treated with vitamin B₁₂ and folic acid, and 2 were treated with additional oral betaine; the methylenetetrahydrofolate-defect patient remained well controlled without betaine.

Between December 1994 and May 1998, blood samples were obtained after an overnight fast and either collected into lithium-heparin tubes and separated within 20 minutes of venesection or collected into EDTA tubes with the plasma separated largely within 1 hour but in some cases, up to 2 hours. In 37 instances, both types of sample were collected in each of the 21 patients. Plasma was divided into aliquots and stored at −70°C until analysis. The study was approved by the Ethics Committee of the University of New South Wales.

Measurements

We measured total plasma homocysteine levels at 2 laboratories: the Cardiovascular Genetics Laboratory, Prince of Wales Hospital (EDTA samples), and the Biochemical Genetics Laboratory, Royal Alexandra Hospital for Children (lithium-heparin samples) by using IMX-automated, fluorescence-based enzyme immunoassays. With this method, total free and protein-bound circulating homocysteine moieties are reduced to free homocysteine with dithiothreitol. The homocysteine is then converted to S-adenosyl-L-homocysteine (SAH) by using SAH hydrolase and excess adenosine. The total homocysteine concentration is determined by fluorescence polarization immunoassay after the addition of an anti-SAHA antibody and a fluoresceinated tracer (S-adenosyl cysteine). The results have been shown to be highly correlated with those obtained by high-performance liquid chromatography (r=0.986). The range of measurement is 0.5 to 50 μmol/L with a sensitivity of <0.5 μmol/L, and with a 1/10 dilution, the assay is linear between 5 and 500 μmol/L.16 The within-assay and between-assay coefficients of variation were 1.9% and 4.1%, and 2.4% and 2.4%, respectively, for the 2 laboratories, as assessed from 2 control samples included in every run of 20 samples. Of our samples, 37 were assayed at both laboratories, and the correlation for homocysteine concentrations from 10 to 250 μmol/L was 0.99. In the lithium-heparin samples, methionine was measured on a Beckman 6300 amino acid analyzer. We measured plasma levels of EC-SOD by ELISA as previously described.17

Statistical Analysis

Because both EC-SOD and homocysteine levels had a skewed distribution, we explored the association between EC-SOD and homocysteine by calculating nonparametric Spearman’s correlation coefficients and the parametric Pearson’s correlation coefficients after the values had been logarithmically transformed. We assessed this relationship by considering the effects of both interindividual and intraindividual variations. For interindividual effects, for each patient we used the first available paired EC-SOD and homocysteine measurement; to include intraindividual variations in the calculations, we used the results of all paired measurements obtained for the patients. Because the distribution of methionine values was also skewed, we assessed the relationships between EC-SOD and methionine and between homocysteine and methionine in the same way.

Results

There were 62 total homocysteine and EC-SOD measurements in the 21 patients. These measurements were taken for blood samples obtained during a period of 3.5 years at various stages of implementation of the treatment regimens. Very high homocysteine levels were available from pretreatment samples in 2 patients with CBS deficiency, and there was an overall wide scatter of levels (range, 10 to 337 μmol/L) as measured by the present method had an R213G mutation, which results in reduced binding affinity to heparin at the endothelial cell surface.15,18 Because it is not known whether this variant is associated with an increased or decreased superoxide-scavenging capability, we excluded this 1 patient from the analysis.

With both the nonparametric Spearman’s test for the original values and the parametric Pearson’s correlation coefficients for the logarithmically transformed data, the EC-SOD and homocysteine levels were found to be highly correlated, for both the first available sample from each
patient and all samples (see Table 1). When a patient had a higher total homocysteine value, ie, at pretreatment or due to a lapse in treatment compliance, the EC-SOD level was also higher. When homocysteine was lower, the EC-SOD was decreased at the same time, although the extent of this lowering varied from individual to individual. When the analyses were confined to the first available paired homocysteine and EC-SOD samples for each patient, the results were the same. For Spearman’s test without transformation and Pearson’s test with logarithmic transformation, the respective correlation coefficients were 0.607 and 0.475 and the probability values, 0.0001 and 0.0001.

The 2 highest homocysteine levels in the series were obtained from CbS-deficient patients at the time of diagnosis and before treatment was commenced. The effects of treatment with oral pyridoxine (100 to 200 mg/d), folic acid (5 mg/d), betaine (6 g/d), and 3 monthly hydrocobalamin injections are shown in Table 2. After treatment, plasma total homocysteine and EC-SOD levels were strikingly decreased while methionine was increased, the latter due to betaine-induced enhanced remethylation of homocysteine to methionine.

In contrast to the positive relationship between EC-SOD and homocysteine, there were no correlations between EC-SOD and the wide range of methionine concentrations as measured by amino acid analysis in the same samples (Table 1). Methionine levels varied between 20 and 1552 \( \mu \text{mol/L} \), the highest levels being in patients receiving oral betaine, and most of the lowest occurred in those with remethylating disorders.

**Discussion**

So far as we are aware, these are the first observations of circulating EC-SOD in patients with homocystinuria due to CbS deficiency or remethylating defects. These measurements have produced an interesting result: the demonstration of a strikingly positive relationship between EC-SOD and homocysteine. With regard to the mechanisms involved, there is evidence for homocysteine-related endothelial dysfunction in homocystinuric patients, which is thought to be due to reduced nitric oxide production. In addition, there is evidence that a mild homocysteine elevation, the so-called hyperhomocysteinemia, also impairs endothelium-mediated vasodilatation and that this event may occur even with a transient homocysteine elevation in response to the challenge of a methionine load. Furthermore, Verhaar and colleagues recently showed that impaired endothelium-dependent vasodilatation in patients with familial hypercholesterolemia can be acutely reversed by the administration of 5-methyltetrahydrofolate, the active form of folate acid. These authors also showed that folate reduced the superoxide generation in in vitro experiments. The administration of 5-methyltetrahydrofolate would be expected to reduce circulating homocysteine, although this parameter was not measured in those experiments. However, a recent study by Usui et al showed no reduction in homocysteine levels after a methionine load when 10 mg of folate acid was given concomitantly, but folate acid did prevent a reduction in the endothelium-dependent vasodilatation, which occurred after a standard oral methionine dose (0.1 g/kg body weight). This phenomenon may represent a counteraction by folate of homocysteine-induced oxidative stress.

The finding of an increase in EC-SOD in association with elevated homocysteine could be a response to homocysteine-induced oxidative damage and could thus constitute a protective mechanism with the effect of opposing oxidative stress. Presumably, elevated levels of homocysteine induce EC-SOD synthesis or the release of EC-SOD into the circulation from the vascular wall. The alternative possibility, that the increased homocysteine may in some way decrease EC-SOD catabolism, seems unlikely. The further possibility that the EC-SOD response is a consequence of folate therapy alone (or indeed, of the other therapies) cannot be excluded by the present data. However, the EC-SOD levels in the treated-patient samples (ie, all but the 2 highest levels; see Table 2) were close to the mean EC-SOD levels that were found in both coronary and noncoronary patients, none of whom was receiving folic acid. Thus, folate lowering of EC-SOD levels seems unlikely.

In the 2 CbS-deficient patients for whom we had measurements before and during treatment, the 50% reduction in plasma EC-SOD that occurred after lowering of the markedly elevated homocysteine levels (Table 2) was documented at 10 weeks and 4 months after starting treatment. We have no data to assess the early time course of the changes.

We have provided evidence suggesting that EC-SOD could have a protective effect in patients with coronary artery disease. In a series of 590 patients aged 65 years with and without coronary artery disease as documented angiographically, EC-SOD levels were lower in patients with a history of acute myocardial infarction than in those without (mean ± SE, 76.1 ± 7.5 versus 110.1 ± 6.0 ng/mL), and a low plasma EC-SOD was independently associated with an increased likelihood of a history of myocardial infarction. Plasma EC-SOD levels were significantly higher in women but lower

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**TABLE 1. Correlation Coefficients (2-Tailed P Values) Between Plasma Levels in All Paired Homocysteine, EC-SOD, and Methionine Samples of 21 Patients**

<table>
<thead>
<tr>
<th></th>
<th>Spearman’s Test</th>
<th>Pearson’s Test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC-SOD vs homocysteine (n=62)</td>
<td>0.607 (0.0001)</td>
<td>0.475 (0.0001)</td>
</tr>
<tr>
<td>EC-SOD vs methionine (n=39)</td>
<td>0.148 (0.368)</td>
<td>0.243 (0.136)</td>
</tr>
<tr>
<td>Homocysteine vs methionine (n=39)</td>
<td>0.321 (0.046)</td>
<td>0.595 (0.0001)</td>
</tr>
</tbody>
</table>

*Pearson’s correlation coefficient was calculated after the levels were logaarithmically transformed.

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**TABLE 2. Effects of Treatment on Plasma EC-SOD, Homocysteine, and Methionine in 2 Newly Diagnosed Unrelated Patients With CbS Deficiency**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Homocysteine, ( \mu \text{mol/L} )</th>
<th>EC-SOD, ng/mL</th>
<th>Methionine, ( \mu \text{mol/L} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>12</td>
<td>337</td>
<td>161.4</td>
<td>699</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Before treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>During treatment*</td>
<td>72.0</td>
<td>71.5</td>
</tr>
<tr>
<td>Patient 2</td>
<td>8</td>
<td>298.3</td>
<td>188.1</td>
<td>581</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Before treatment</td>
<td>50.1</td>
<td>98.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>During treatment</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Both patients were treated with daily oral doses of pyridoxine 100–200 mg, folic acid 5 mg, and betaine 6 g; see text. Posttreatment measurements in patient 1 were made 4 months after starting treatment, and in patient 2, 10 weeks.
in smokers of both sexes within the normal range. All of these results are consistent with the concept of an increase in circulating EC-SOD levels being associated with reduced cardiovascular risk. Extrapolating from these findings, the EC-SOD increase that we have identified in homocystinuric patients in relation to a broad range of increased homocysteine should be helpful in diminishing the risk of vascular damage in this high-vascular-risk population with greatly increased homocysteine levels. None of the patients in the present study have had a vascular event. The relation between elevated total homocysteine levels and vascular risk is likely to be complex and may be attenuated at higher levels (perhaps >30 μmol/L) because of enhanced EC-SOD production. This could explain the paradox that mildly elevated levels of homocysteine are associated with an increased vascular risk but that the much higher levels of homocysteine seen in patients with treated CBS deficiency are apparently not associated with a concomitantly much higher risk. There remains the possibility of an independent effect of folic acid therapy, which all treated patients were receiving.

In conclusion, patients with significantly elevated homocysteine due to CBS deficiency also have elevated levels of EC-SOD, which decline when the homocysteine levels are lowered. This EC-SOD response may be protective and may partly explain why the apparent risk of a vascular event in treated homocystinuric patients is not extremely high, even though their homocysteine levels remain several times greater than those usually seen in the mild hyperhomocysteinemia associated with common forms of vascular disease.

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